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2-Morpholinoethylisocyanide as a coupling agent for hapten-protein conjugates

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Abstract
Conjugation of haptens to proteins and enzymes is essential for the production of antibodies and development of ELISAs for these low molecular weight compounds. In this study, protein conjugates of several carboxylic haptens were prepared using the peptide synthesis reagent, 2-morpholinoethylisocyanide. The haptens included derivatives of benzimidazole and pyridoxine. The main advantage of this conjugation procedure is its simplicity involving only small volumes of water-miscible organic reagents. The resulting conjugates were purified by dialysis and could be used to elicit antibodies and establish immunoassays.

Keywords: *Hapten, antibodies, benzimidazole.*

Introduction

The coupling of a low molecular weight organic compound, or hapten, to a protein carrier is a necessary first step in developing an immunoassay for that compound, and even the immunogenicity of peptides is enhanced by conjugation to a macromolecular carrier (Kirkley et al. 2001). Hapten coupling can be achieved by a variety of reactions and can exploit an existing functional group or an added reactive group (Erlanger 1980, Brinkley 1992, Wong 1991). Among the coupling methods available, one of the most widely used procedures is the induction of amide bond formation using carbodiimides, reagents originally applied to peptide synthesis (Rich & Singh 1979, Williams & Ibrahim 1981). Water-soluble carbodiimides enable protein-hapten conjugate formation in aqueous solvents and have been widely used for synthesis of immunogenic conjugates (for example, Bauminger & Wilchek 1980, Fuentes et al. 2005) and, to a limited extent, enzyme conjugates (Avrameas et al. 1978). The utility of water-soluble carbodiimides is limited by the sparing solubility of some haptens in water and by the formation of the intermediate O-acylisourea derivative and N-acylurea side products, which are typically less soluble than the parent molecule. The mixed anhydride method, using an alkylchlorocarbonate reagent, can also result in conjugation of the alkylcarbonate component (Gendloff et al. 1986) that, like stable acylisourea side products, could act as unwanted haptens.

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Isocyanides were developed as reagents for immobilization of biological molecules, with retention of activity (Axen et al. 1971, Vretblad & Axen 1973, Drevin & Richter 1985). These reactions were adapted and modified to provide an alternative to carbodiimides for peptide synthesis (Aigner et al. 1982). Isocyanides with tertiary amino groups, such as 2-morpholinoethylisocyanide (MEI), in the presence of a suitable additive such as N-hydroxysuccinimide (NHS), promoted peptide synthesis in high yield. We previously adapted the MEI peptide synthesis procedures to prepare hapten conjugates of zeatin and benzimidazole derivatives (Brandon & Corse 1993, Brandon et al. 1994). In this study, we investigated the use of MEI for coupling additional haptens to proteins, as exemplified in the development of two ELISA formats for mebendazole.

Materials and methods

Chemicals and immunochemicals

Bovine serum albumin (BSA) was obtained from Miles (Elkhart, IN, USA) or Serologicals (Purchase, NY, USA). Keyhole limpet hemocyanin (KLH) was from Calbiochem (La Jolla, CA, USA). Horseradish peroxidase (HRP), ovalbumin (OVA), and (S)-(-)-α-amino-γ-butyrolactone hydrobromide were obtained from Sigma-Aldrich (St Louis, MO, USA). HRP-labeled rabbit anti-mouse IgG was obtained from Zymed Laboratories (South San Francisco, CA, USA). Mebendazole (MBZ) and its *p*-fluoro derivative, flubendazole, were purchased from Janssen Pharmaceutical (Piscataway, NJ, USA) and 2-morpholinoethylisocyanide was obtained from Fluka (Ronkonkoma, NY, USA). All other commercial chemicals were reagent grade products. Pyridoxylacetic acid (PAA) was synthesized by the method of Tomita et al. (1967), and the structure was confirmed by NMR.

^{1}H NMR (DMSO-d6): δ 7.90 (s, 1H), 5.40 (s, 2H), 3.73 (s, 2H), 2.40 (s, 3H). 2-Succinamidomebendazole (succinamido-MBZ) was synthesized as follows: 4-Amino-3-nitrobenzophenone was synthesized by heating 4-chloro-3-nitrobenzophenone with tetramethylene sulfone (sulfolane), propanol and NH_4OH at 125°C for 18 h in a bomb. Solvent was removed on a rotary evaporator to give a mixture of product plus sulfolane. After acidification and heating on a steam bath, sulfolane was removed. The product was crystallized from toluene, catalytically hydrogenated to form 3,4-diaminobenzophenone, and converted to 5-benzoyl-2-aminobenzimidazole by reaction with CNBr. The aminobenzimidazole derivative was then reacted with succinic anhydride in dry pyridine. Following purification by a series of precipitations, the 2-succinamido-mebendazole hapten was obtained, and the purity was confirmed by NMR.

^{1}H NMR (400 MHz, DMSO-d6): δ 2.50 (1H, s, NH), 2.59 (2H, t, CH2), 2.72 (2H, t, CH2), 7.54–7.73 (aromatic H), 12.07 (1H,d, NH). 13C NMR δ 195.5, 173.5 (2C), 171.7 (2C), 138.4 (2C), 131.7, 129.2 (3C), 128.2 (4C), 123.6, 30.4, 28.4.

N-carboxypentanoylhomoserine lactone was synthesized as follows. Three mmol (546 mg) (S)-(-)-α-amino-γ-butyrolactone hydrobromide was mixed with a solution of 309 mg $KHCO_3$ in 1.2 ml water. CH_2Cl_2 was added and most of the water was removed from the mixture by addition of anhydrous Na_2SO_4 and molecular sieves. After separation of the solution, the solids were extracted with ethyl acetate. This solution was also dried. Adipic acid (5.95 mmol, 870 mg) was dissolved in 5 ml

tetrahydrofuran and 270 ml oxalyl chloride was added. A precipitate was filtered out and the CH_2Cl_2 and ethyl acetate solutions were added to the filtrate. The mixture was stirred and warmed to boil out some of the CH_2Cl_2, after which 0.4 ml dry triethylamine was added. This mixture was filtered and solvent was removed from the filtrate on a rotary evaporator to give an 855 mg residue. The residue was taken up in tetrahydrofuran and filtered. Attempts to obtain crystals were unsuccessful. The solvent was removed to give an 800 mg residue, which was extracted with ether. The remaining solid (450 mg) was analysed and determined to be the target compound $C_{10}H_{15}NO_5$ (M_r 229). LC/MS spectra using atmospheric pressure chemical injection and negative ion electrospray showed peaks for m/z 230 and 228.1, respectively.

^{13}C NMR (methanol-d4): δ 177.4(2C), 176.0, 67.2, 47.9, 36.4, 34.7, 29.7, 26.2, 25.6.

Conjugation of pyridoxylacetic acid. OVA and BSA were dialyzed against distilled water and concentrations were determined spectrophotometrically, using $A_{280, 1 g l^{-1}} = 0.65$ for OVA and 0.69 for BSA. In a typical conjugation reaction, 110 mg PAA (0.5 mmol) dissolved in 0.7 ml dimethylformamide (DMF) was mixed with 1 mmol hydroxybenzotriazole (HBT) or NHS dissolved in 0.5 ml DMF and 1.2 mmol (52 μl) MEI. The reactants were stirred for 30 min at room temperature in an amber vial, and then 1 mmol dimethylaminopentane or triethylamine (TEA) in 67% DMF in water (v/v) was added. The mixture was added dropwise to an aqueous solution of carrier protein (4 ml, 2.5 g l^{-1}), and then stirred for 16 h at ambient temperature in the dark. The reaction mixture was then dialyzed against phosphate-buffered saline (PBS, 150 mM NaCl, 5 mM Na phosphate, pH 7.0) containing 0.1 mg l^{-1} NaN$_3$ to inhibit microbial growth. Control reactions were performed without MEI or with acetic acid substituted for PAA.

N-acylhomoserine lactone hapten conjugation to BSA and KLH. N-Carboxypentanoyl-homoserine lactone (45 μmol in 0.3 ml DMF) was combined with an equimolar amount of HBT and a 20% molar excess of MEI and incubated for 30 min. An equimolar amount of TEA was added as a 10% solution in DMF. The resulting mixture was added dropwise, with stirring, to 20 mg BSA in 1 ml water or to 20 mg KLH in 4 ml of 0.4 M NaCl. The mixtures were incubated 16 h at ambient temperature and then dialyzed exhaustively against 0.3 M NaCl. Samples were then analyzed for free amino groups.

Analysis of hapten conjugates. The following extinction coefficients were used to analyse conjugates by UV-visible spectrometry: PAA, $A_{330, 1 mM} = 5.7$; succinamido-MBZ, $A_{310, 1 mM} = 12$. Protein content was estimated by the bicinchoninic acid procedure (Smith et al. 1985) or by Coomassie Blue dye binding (Bradford 1976). For haptens lacking a chromophore, degree of conjugation was estimated from the change in free amino groups determined using trinitrobenzenesulfonic acid (TNBS), essentially according to Habeeb (1966). Reaction mixtures of 0.2 ml (sample in 45 mM NaHCO$_3$, pH 8.5, plus TNBS, 3.4 μM) were incubated 2 h at 37°C. Sodium dodecylsulfate (0.2 ml, 100 g l^{-1}) and 0.1 ml 1 N HCl were then added, and the absorbance at 340 nm was determined. Calculations were based on $E_{mM} = 10$ for the trinitrophenyl chromophore. Some conjugates were also analyzed using sinipinic acid matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass

spectrometry. Samples were run in linear mode on a Reflex II mass spectrometer equipped with a 337 nm UV laser and XMASS software (Bruker, Billerica, MA, USA).

Conjugates of succinamido-MBZ. Conjugates of BSA and HRP were prepared as for PAA (above). The enzyme conjugate was stored at 4°C at an HRP concentration of 1 g l^{-1} in PBS containing 10 g l^{-1} BSA and 0.2 mg l^{-1} thimerosal.

Preparation of hapten-specific antibodies. Preparation of mouse monoclonal antibodies (mAbs) elicited with the 2-succinamido-MBZ conjugate was performed as described previously (Brandon et al. 1992).

ELISA methods

(1) Preparation of assay plates. Antibody or hapten conjugate was dissolved at 5 mg l^{-1} in PBS and coated on microtitration plates by incubation (100 µl/well) for 4 h at room temperature or 16 h at 4°C, followed by rinsing 5 times with distilled water. Uncoated "sticky" sites on wells were blocked by incubation for 1 h at room temperature with PBS containing 10 g l^{-1} bovine serum albumin and 0.02% (v/v) Tween-20 (BSA-PBS-Tween, 200 µl/well). The plates were again washed 5 times with water. Plates were stored with wells filled with PBS+NaN$_3$ at 4°C for up to 4 weeks. Alternatively, washed and drained plates were incubated with 20 g l^{-1} sucrose (200 µl/well) for 30 min, then drained and dried at 37°C for 1 h. Dried plates were stored desiccated at 4°C for up to 6 months.

(2) Competitive ELISAs for MBZ. In Format 1, polystyrene assay wells were coated with succinamido-MBZ-BSA. Standard samples were premixed with an equal volume of purified IgG anti-MBZ from hybridoma clone 525 (mAb 525, 5 µg l^{-1}) and applied to the assay wells (100 µl/well) for 1 h, with shaking. Unbound antibody was removed by washing 5 times with water. HRP-rabbit anti-mouse IgG (1:1000 dilution from commercial stock solution, 100 µl/well) was added to the wells and incubated for 1 h. Unbound conjugate was removed by washing the plates 5 times with water, and bound HRP was visualized using tetramethylbenzidine substrate solution (TMB; K-Blue Substrate, Neogen, Lexington, KY, USA; 100 µl/well; 15 min) and 0.3 N HCl to stop the reaction (100 µl/well). Absorbances were determined using a Vmax plate reader (Molecular Devices, Sunnyvale, CA, USA). In Format 2, polystyrene assay plates were coated with purified mAb 525 IgG. Standard samples were premixed with an equal volume of succinamido-MBZ-HRP (0.75 mg l^{-1} in BSA-PBS-Tween) and incubated in the assay wells for 1 h (100 µl/well), with shaking. Unbound HRP conjugate was removed by washing the plates 5 times with water, and the assay was developed as in Format 1.

Curve fitting. Fitted curves were computed using SlideWrite Plus (Advanced Graphics Software, Encinitas, CA, USA).

Results

The structures of the haptens used in this study are shown in Figure 1 and the overall reaction scheme is shown in Figure 2. Table I shows the results of preparing
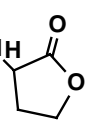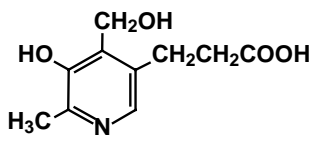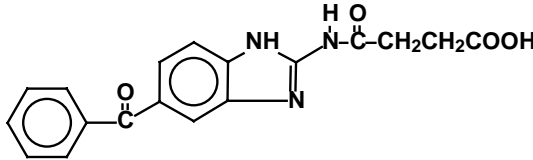
Figure 1. Haptens conjugated to proteins using 2-morpholinoethylisocyanide.

conjugates of bovine serum albumin and ovalbumin with pyridoxylacetic acid. In these experiments, the molar ratio of reactants (carboxyl compound:additive:tertiary amine:MEI, 1:1:1:1.2) was that used by Aigner et al. (1982). Table I shows that
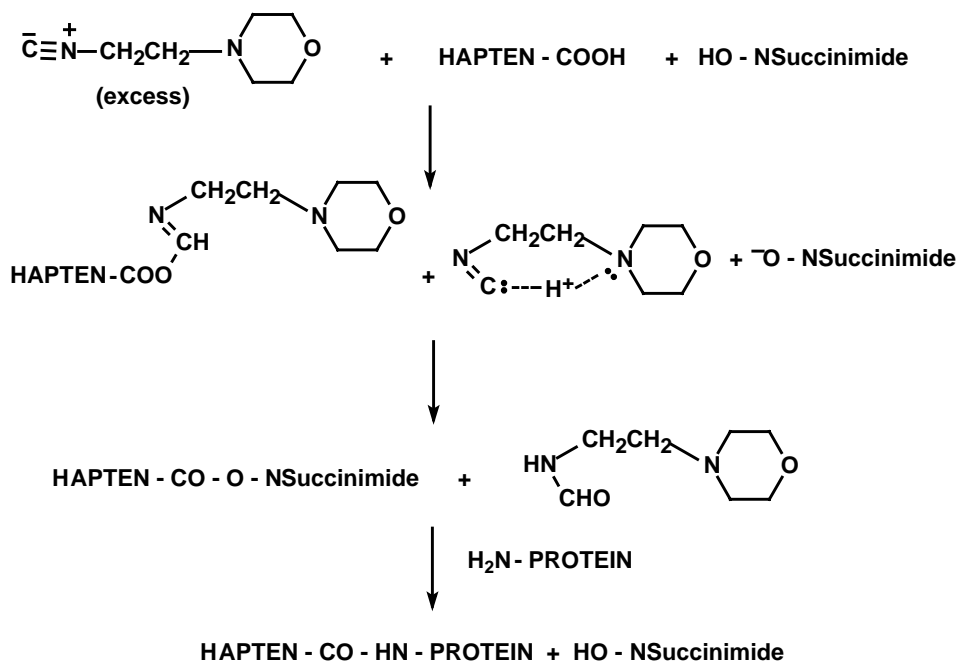

Figure 2. Reaction scheme for hapten-protein coupling using 2-morpholinoethylisocyanide (based on Aigner et al. 1982).

Table I. Conjugation of pyridoxylacetic acid to bovine serum albumin and ovalbumin.

Protein	Hapten	Conjugation ratio[b]
BSA[a]	–	0
BSA	Acetic acid	0
BSA	PAA	26 ± 6
OVA[a]	–	0
OVA	Acetic acid	0
OVA	PAA	14 ± 1

[a]Controls, without hapten, MEI, or additives (hydroxybenzotriazole or N-hydroxysuccinimide); [b]mol PAA/mol protein determined spectrophotometrically.

both proteins were heavily conjugated under these conditions: one-half of the ε-amino groups of BSA were derivatized and 70% of those of OVA.

Table II shows the results of two conjugation reactions with the homoserine lactone hapten. Because this hapten lacks a chromophore, the degree of conjugation was estimated from the decrease of free amino groups in the carrier protein. A high apparent degree of conjugation was achieved, with over 80% of the available amino groups of BSA blocked by the conjugation reaction. The KLH conjugate had about 20% of its amino groups blocked in the reaction, based on the content of 38 mol amino groups/100,000 g protein for mollusc hemocyanins (Waxman 1975). In preliminary studies, use of water as solvent for KLH resulted in protein precipitation that did not occur with BSA or OVA. The use of 0.4 M NaCl as solvent for KLH (see Methods) eliminated this problem. The BSA conjugate and control were also analyzed by MALDI-TOF mass spectrometry (Table II), and results indicated a conjugation ratio of 14 mol hapten/mol BSA. Thus, conjugated homoserine lactone accounted for about one-third of the amino groups that became unavailable to react with TNBS.

Using the standard conjugation conditions, succinamido-MBZ was conjugated with BSA at 3.5 mol/mol protein and with HRP at 2.6 mol hapten/mol enzyme, determined spectrophotometrically. Using the BSA conjugate, antibodies were elicited in BALB/c mice, hybridomas were produced and screened by ELISA. Cells producing candidate MBZ-specific antibodies were cloned and recloned before the mAbs were characterized. Monoclonal Ab 525 was selected for purification and further analysis using two ELISA formats, as shown in Figure 3. Both formats gave comparable sensitivities, with the midpoint of the assay response curve (IC_{50}) at 4.4 ppb mebendazole. The working ranges for the assays (estimated as IC_{20} to IC_{80}) were about 1–20 ppb (Format 1) and 2–10 ppb (Format 2). In both ELISAs, mAb 525 exhibited 10%

Table II. Conjugation of homoserine lactone hapten.

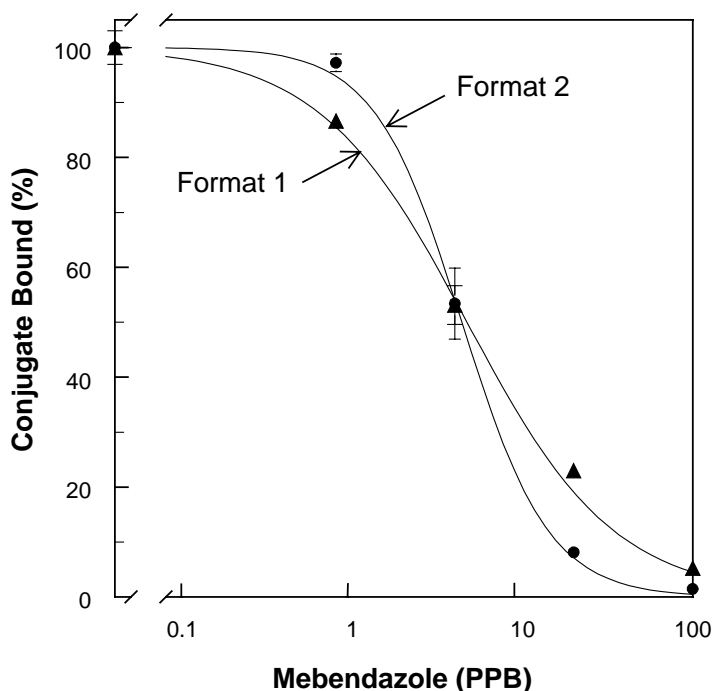| Sample	Conjugation ratio[a]	
	Calculated from amino groups	Determined by MALDI-TOF mass spectrometry
BSA conjugate	46 ± 2	18
BSA control	1.6 ± 0.2	0
KLH conjugate	10 ± 1	not done
KLH control	0 ± 3	not done

[a]mol/mol BSA or mol/100,000 Da KLH.

Figure 3. Standard curves for mebendazole in two ELISA formats: Format 1, succinamido-MBZ-BSA conjugate-coated wells, with HRP-conjugated rabbit anti-mouse IgG to detect bound mAb 525 (▲); Format 2, mAb 525 IgG-coated assay wells, with succinamido-MBZ-HRP conjugate as labeled ligand (●).

cross-reactivity with flubendazole, the *p*-fluoro analog of mebendazole, but essentially no cross-reactivity with other benzimidazoles tested.

Discussion

A desirable coupling reaction must employ conditions that do not denature proteins and enzymes. In addition, it should be adaptable to a micro scale and permit a simple procedure for purification, such as dialysis. The procedure using MEI, as described above, meets these requirements. A particularly useful feature is that solvent conditions can be modified for selected proteins or for more hydrophobic haptens. We have used either 100% DMF or various concentrations of DMSO in DMF to maintain the solubility of the hapten (before and during its activation by MEI). In addition, highly soluble proteins like BSA can be dissolved in 25–50% (v/v) DMSO in water. The water-miscible organic solvent prevented precipitation of activated MBZ hapten, for example. Analyses of hapten conjugates by mass spectroscopy as well as gel electrophoresis are consistent with the formation of some protein oligomers by conjugations using either carbodiimides (Singh et al. 2004) or MEI (Brandon, unpublished observations). Varying the solvent conditions could possibly influence the degree of unwanted protein-protein cross-linking that occurs during hapten conjugations.

The analysis of the homoserine lactone conjugate of BSA indicated that about half of the total amino groups had become unavailable to react with TNBS *without*

apparent incorporation of the hapten. Similar effects have been noted with carbodiimide coupling of some haptens (Adamczyk et al. 1994), and could be due to changes in tertiary structure, aggregation, crosslinking of the carrier or the formation of non-covalent adducts, N-acyl urea adducts (for carbodiimides) or uncharacterized side reactions.

The hapten density of protein conjugates is known to affect their immunogenic properties, with relatively low densities favoring a high affinity, predominantly IgG response (Klaus & Cross 1974), owing to the relative strength of interactions with T- and B- cell antigen receptors. In contrast, using a fusion protein with multiple, repeating peptide epitopes, Liu and Chen (2005) found that a high epitope density enhanced the production of high affinity rabbit antibodies. Singh et al. (2004) reported 2- to 3-fold higher titers of rabbit antibodies in response to highly conjugated BSA (14–17 mol/mol) compared to low epitope densities (3–10 mol/mol). The plasma clearance rates of hapten-protein conjugates are also sensitive to the epitope density (Park et al. 1987), providing an additional influence on the immune response to hapten conjugates. The immunizing conjugate may thus affect the quantity, quality, and utility of resulting hapten-specific antibodies in ELISA and other applications (Marco et al. 1995). In addition, a low hapten density of enzyme conjugates in ELISA could help to avoid inactivation of the enzyme or its high avidity binding to immobilized antibody, either of which could lower assay sensitivity. The coupling procedure utilizing MEI typically yields conjugation ratios of 4-18 mol/mol BSA, providing both effective immunogens and useful plate-coating conjugates, and 2–5 mol/mol HRP for highly active hapten-enzyme conjugates. In our experience, the degree of conjugation is sensitive to the ratio of MEI-activated hapten to protein and could be used to vary the hapten density of conjugates.

Acknowledgements

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